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RecA protein filaments can juxtapose DNA ends: An activity that may reflect a function in DNA repair

(electron microscopy/DNA joining)

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ABSTRACT **To further characterize the role of RecA protein–DNA filaments in general recombination and DNA repair, we have examined interactions of these filaments with themselves following formation. When linear double-stranded DNA was incubated with RecA in the presence of Mg^{2+} and adenosine 5′-[γ-thio]triphosphate, monomer-length (1*n*) nucleoprotein filaments were observed. Following continued incubation, filaments having 2*n*, 3*n*, . . . lengths were observed, indicating that an end-to-end joining of the monomer-length filaments had occurred. When linear single-stranded DNA was covered by RecA protein under several conditions, the ends of the resulting filaments joined together rapidly, producing circular filaments. The end-to-end joining of single-stranded DNA–RecA filaments appeared to require that 3′ DNA ends be juxtaposed with 5′ DNA ends, because double-stranded DNA molecules having long single-stranded DNA tails with only 3′ or 5′ termini did not join end-to-end. However, when both 5′ and 3′ ends were present in the reaction, joining was observed. We suggest that this end-to-end joining activity may help explain the role of RecA protein in both the protection of damaged DNA ends and the repair of double-stranded DNA breaks.**

One of the most fascinating properties of the RecA protein of *Escherichia coli* is its ability to form filaments. Ultrastructurally similar filaments are formed by RecA protein along single-stranded DNA (ssDNA; refs. 1–3), double-stranded DNA (dsDNA; refs. 1, 2, 4), RNA (5), and even in the absence of polynucleotides (1, 6–8). RecA–nucleic acid filaments will stimulate repressor cleavage (9) and initiate strand exchange *in vitro* (for review, see ref. 10), and it has been proposed that they function similarly in the cell. Griffith and Formosa (11) have proposed that RecA protein is a prototype for a class of DNA binding proteins that they termed "recombinational scaffolding proteins." We contend that these proteins must bind to DNA in an ordered and cooperative manner to be active. If this is true, the most relevant functional studies of these proteins will be of their filamentous forms.

In addition to controlling general recombination and the SOS response, genetic evidence indicates that RecA protein is involved in several poorly understood facets of DNA repair. These include protection of DNA ends after various types of damage (12) and repair of dsDNA breaks (13). Although the molecular bases of these processes remain unknown, it seems likely that they involve the formation of protein filaments along the damaged DNA. If so, properties of RecA–DNA filaments must be identified that might facilitate these repair functions.

In this paper, we describe conditions under which RecA protein filaments containing linear DNA can join end-to-end, juxtaposing the DNA ends. We have termed this activity "end-joining." This property might play several roles in DNA repair, including properly positioning severed dsDNA ends prior to repair and protecting damaged DNA ends from degradation.

MATERIALS AND METHODS

Proteins. RecA was purified from *E. coli* overproducer strain KM4104/pDR1453 as described (14). SSB (single-strand binding protein) was purified as described (15) from an *E. coli* overproducer strain (gift of J. Chase). *E. coli* exonuclease III (16) and phage T4 ligase (17) were purified as described. RecA and SSB concentrations were determined by the method of Bradford (18).

DNA. Plasmids pBR322 and pUR222 and phage M13 dsDNA were amplified by chloramphenicol treatment in *E. coli* HB101(pBR322, pUR222) or K37(M13). The DNA was purified by the method of Modrich and Zabel (19). Both pBR322 and pUR222 DNAs were linearized by *Eco*RI (the restriction enzyme was a gift of P. Modrich). Plasmid pUR222 DNA was also cleaved by *Sal* I (Bethesda Research Laboratories), *Pst* I (Bethesda Research Laboratories), and *Hin*dII (Boehringer Mannheim). M13 dsDNA was cut by *Hin*cII (Bethesda Research Laboratories). Linear M13 dsDNA (40 μg/ml) was digested by phage λ exonuclease (New England Biolabs) in 100 mM glycine, pH 9.4/3 mM $MgCl_2$/3 mM 2-mercaptoethanol, using 150 units of enzyme/ml for 20 min at 21°C. Exonuclease III digestion of linear M13 dsDNA (40 μg/ml) was carried out in 50 mM Tris·HCl, pH 8.0/1 mM $MgCl_2$/1 mM 2-mercaptoethanol, using 150 units of enzyme/ml for 20 min at 37°C. Tails generated by either exonuclease were determined by electron microscopy to be 1000–3000 bases long. M13 mp7 ssDNA was purified from phage by treatment with chloroform and 0.5% *N*-dodecanoylsarcosine followed by equilibrium density banding in CsCl. M13 mp7 ssDNA contains a duplex stem with an *Eco*RI restriction site. Addition of 1 unit of *Eco*RI/μg of ssDNA followed by incubation at 37°C for 45 min yielded a population of >99.5% linear molecules.

Assembly of RecA Protein onto DNA. RecA was incubated with dsDNA at a molar ratio of 1 RecA monomer per 2.5 base pairs of DNA (a protein/DNA mass ratio of 20:1; ref. 20) in 20 mM Hepes, pH 7.5/2 mM $MgCl_2$/0.6 mM adenosine 5′-[γ-thio]triphosphate (ATP[γ-S]) at 37°C for various times. M13 mp7 ssDNA (9.2 nmol of base/ml), or M13 dsDNA that had been treated with either λ exonuclease or exonuclease III (to give 9.2 nmol of single-strand base/ml), was incubated with RecA protein (at a molar ratio of 1 RecA monomer per 3 bases of DNA) in 30 mM NaCl/3 mM ATP or dATP (or 0.6 mM ATP[γ-S])/12 mM $MgCl_2$ or $MnCl_2$/20 mM Hepes, pH 7.5, at 37°C for various times. An ATP-regenerating system

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Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; SSB, single-strand binding protein; ATP[γ-S], adenosine 5′-[γ-thio]triphosphate.

consisting of creatine phosphokinase (4 μg/ml; Sigma) and phosphocreatine (20 mM; Sigma) was included in all reactions except those where ATP[γ-S] was used. When SSB protein was used, it was added at a molar ratio of 1 SSB per 18.2 bases of ssDNA (a 3:1 protein/DNA mass ratio) 5 min after addition of the RecA protein to the ssDNA.

Ligation of DNA in End-Joined Filaments. End-joined filaments were formed as described above and in the legends to Tables 1 and 2. The buffers were then adjusted to 50 mM $MgCl_2$, 20 mM dithiothreitol, 1.0 mM ATP, and 50 μg of bovine serum albumin/ml (for reactions using phage T4 DNA ligase) or 4 mM $MgCl_2$, 1.0 mM dithiothreitol, 26 μM NAD (Sigma), and 50 μg of bovine serum albumin/ml (for reactions using *E. coli* DNA ligase) to accommodate the ligases. The stability of the end-joined filaments 30 min after these adjustments were made was verified by electron microscopy. Either phage T4 or *E. coli* DNA ligase (New England Biolabs) was added at from 10 to 100 units/ml to reaction mixtures containing from 5 to 200 μg of DNA/ml. Incubations were from 3 to 16 hr at either 16°C or 37°C. Ligation of filaments containing dsDNA was assayed by agarose gel electrophoresis following deproteinization with 0.1% sodium dodecyl sulfate (21). Ligation of filaments containing ssDNA was assayed by surface-spreading the deproteinized DNA in 40% (vol/vol) formamide.

Electron Microscopy. DNA–protein complexes were fixed, mounted onto thin carbon films, and rotary shadow-cast with tungsten (20, 22). Micrographs were taken on a Philips EM400 TLG, and length measurements were made by directly projecting 35-mm micrographs onto a Summagraphics digitizer coupled to an Apple II computer.

RESULTS AND DISCUSSION

RecA Protein Filaments Can Juxtapose dsDNA Ends. The binding of RecA to dsDNA in the presence of Mg^{2+} and ATP[γ-S] produces stable nucleoprotein filaments with an 8.5-nm helical repeat (1, 2, 4, 6). In these stiff filaments, the protein-bound DNA is unwound by 11.5°/base pair and is extended 1.6-fold (1, 2, 4, 20, 23). When incubations of RecA with dsDNA were carried out under these conditions for 1 hr, all the DNA became complexed into monomer-length filaments. Upon continued incubation, however, a large percentage of the filaments had lengths that were integral multiples of the unit length (Fig. 1 *A* and *B*; Table 1), indicating that the monomer-length filaments had joined end-to-end. As the concentration of DNA ends was increased from 2.6 pmol/ml to 104 pmol/ml, the percentage of end-joined filaments increased (Table 1).

Only intermolecular end-joining of filaments containing dsDNA was observed in these experiments, even when the concentration of DNA ends was as low as 0.1 pmol/ml. This was probably due to the rigid nature of these filaments. Although the DNA used in the experiment shown in Fig. 1 *A* and *B* contained complementary ends, end-joining did not require base-pairing of the DNA. We compared the end-joining of filaments containing linear pUR222 DNA produced by cleavage by either *Eco*RI (which generates 4-base 5′ overhangs), *Pst* I (which generates 4-base 3′ overhangs), or *Hin*dII (which generates blunt ends). End-joining of all templates was seen, although a higher level was observed if the DNA contained complementary ends (Table 1). This higher level was not due to annealing of the ends prior to protein coverage, because monomer-length filaments were precursors to the multimers; nor was it due to the single-stranded nature of the ends, as almost no end-joining was seen between filaments containing DNA with noncomplementary ends (Table 2). Instead, it is more likely that complementary ends stabilize the joints following alignment of the filaments. The lack of end-joining observed when the DNA contained noncomplementary ends was probably due to a local disruption of the protein helix and/or repulsion between unmatched bases, resulting in an unstable joint.

RecA Filaments Can Juxtapose ssDNA Ends. RecA has been shown to assemble into stable filaments along ssDNA in the presence of Mg^{2+}, ATP, and SSB protein (24, 25). As visualized by electron microscopy, this assembly occurs in three steps (24). First, SSB rapidly covers the ssDNA. RecA then nucleates onto the SSB-covered ssDNA. Finally, additional RecA cooperatively polymerizes 5′ to 3′ (26) onto the ssDNA, displacing the SSB. To determine whether linear RecA–ssDNA filaments could end-join, RecA was assembled onto linear M13 mp7 ssDNA and samples were examined by electron microscopy. After incubations of 10–20 min, 5–25% of the filaments observed were monomer-length circles (Fig. 1*C*), indicating that intramolecular end-joining had occurred. The percentage of circular filaments increased to 20–50% following incubations of 40–50 min (Table 3).

In these reactions, circularization appeared to occur rapidly after complete coverage with RecA protein, as <20% of the fully RecA-covered filaments were linear. It also appeared that only filaments containing ssDNA in which the SSB protein had been completely displaced by RecA were end-joined, as none of the circular filaments contained visible tracts of SSB. Thus, it appears that the increase in the fraction of circular filaments obtained with continued incubation was governed more by the rate at which both ends of the DNA became covered by RecA protein than by the rate of end-joining. Aggregation of the complexes at DNA concentrations >150 nmol of base/ml (50 μg/ml) made reproducible electron microscopic examination impossible at these concentrations.

When circular filaments containing linear ssDNA form, the 5′ end of the ssDNA is juxtaposed with the 3′ end. To determine whether 3′ ends could be juxtaposed with 3′ ends, we assembled RecA protein onto long ssDNA tails that had been generated by λ exonuclease treatment of linear M13 dsDNA. Following incubations of 15–60 min, no circles or linear multimers were observed. There was also no end-joining observed when the ssDNA tails had 5′ termini (produced by exonuclease III treatment). However, when RecA protein was assembled onto a mixture of these two DNA species, double-length molecules were observed (2–10%) which contained two dsDNA segments joined by a long RecA protein-covered segment (Fig. 1*D*). We have reported that RecA protein assembles 5′→3′ along ssDNA (26), and it appears that like this assembly, end-joining of these filaments is an ordered reaction; 3′ ends can be juxtaposed only with 5′ ends. If RecA is arranged in a helix along ssDNA, these results argue that for end-joining to occur, the direction of coiling in the helix must be maintained across the joint.

A variety of conditions that have been shown to stimulate the binding of RecA to ssDNA (6) were examined for their ability to promote end-joining of these complexes (Table 3). Although end-joining was observed under all conditions examined, two results deserve further comment: (*i*) Approximately the same level of end-joining was obtained in the presence of ATP or dATP, but much less was observed when Mn^{2+} was present rather than Mg^{2+}. This correlates well with the low percentage of molecules that were fully covered by RecA protein when Mn^{2+} was used in place of Mg^{2+} (Table 3). (*ii*) Slightly higher levels of end-joining were observed in the absence of SSB protein than in its presence. This was probably due to a combination of two factors: (*a*) RecA binds cooperatively to ssDNA even in the absence of SSB (1–3), and (*b*) in the presence of SSB, a majority of filaments have a short segment of SSB protein at the 5′ ends, which appears to block end-joining.

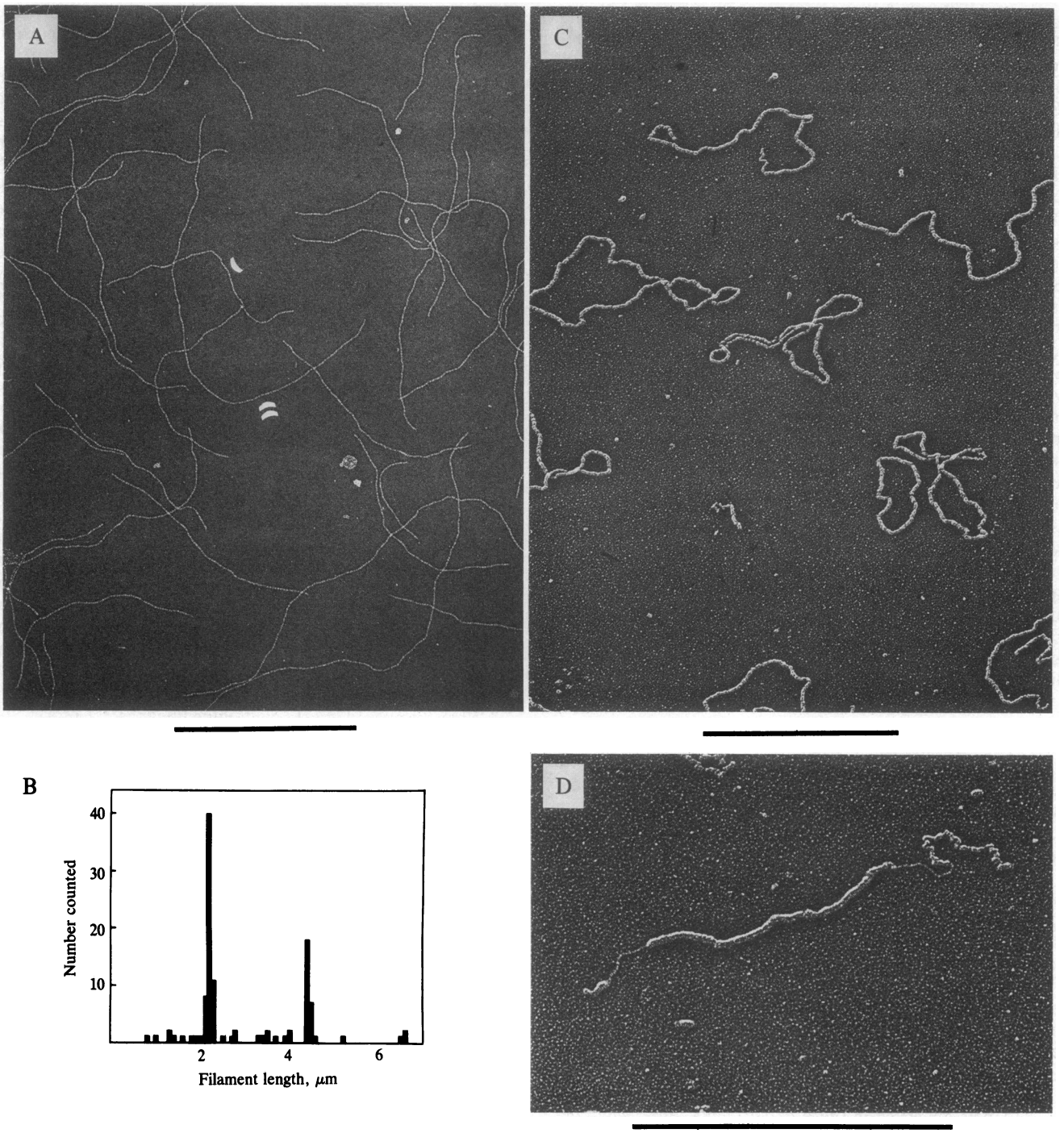

FIG. 1. Electron microscopic visualization of end-joined filaments. (*A*) RecA was incubated with linear pBR322 dsDNA in the presence of 2 mM $MgCl_2$ and 0.6 mM ATP[γ-S] for 3 hr at 37°C to form end-joined filaments. The single arrowhead indicates a monomer-length filament; the double arrowhead indicates a double-length filament. (Bar = 1 μm.) (*B*) Random fields of molecules such as *A* were photographed and the filament lengths were measured. Monomer-length filaments are 2.1 μm long. (*C* and *D*) RecA was incubated with either linear M13 mp7 ssDNA (*C*) or a mixture of dsDNA containing long ssDNA tails with 5′ or 3′ ends (*D*) in the presence of Mg^{2+}, ATP, and SSB protein for 45 min at 37°C to generate end-joined filaments. *D* shows two linear dsDNA molecules with long ssDNA tails that have been juxtaposed by the RecA protein filaments that formed along their ssDNA tails. Micrographs *C* and *D* were taken at a 45° tilt to increase contrast; therefore magnifications are only approximate. (Bars in *C* and *D* ≈ 0.3 μm.)

End-Joining of Filaments Containing RNA. We previously reported that RecA protein forms filaments when it binds to RNA in the presence of Mg^{2+} and ATP[γ-S] (5). In that study, very long filaments were observed, which contained a large amount of low molecular weight poly(A). From the results presented here, it appears that these filaments were the product of end-joining. Further investigation has revealed that in the presence of Mg^{2+} and ATP[γ-S], RecA binds poorly to natural RNA as contrasted to ribohomopolymers. Thus, no end-joining of natural RNA by RecA protein was observed (data not shown). The low level of filament-formation along RNA is probably due to a low affinity of RecA for RNA (6, 27) and the inability of RecA to completely disrupt the secondary structure present in natural RNA.

Possible *in Vivo* Roles for DNA End-Joining in *E. coli*. Two possibilities merit consideration.

(*i*) End-joining of DNA could play a role in the repair of dsDNA breaks. Although little is known about the molecular

Table 1. End-joining of filaments containing dsDNA

DNA ends		% filaments				
Structure	Conc., pmol/ml	1*n*	2*n*	3*n*	4*n*	>4*n*
4-Base 5′ overhang	2.6	77	23			
	5.2	54	46			
	10.4	44	56			
4-Base 3′ overhang	2.6	86	14			
	5.2	78	22			
	10.4	66	34			
	26	21	18	27	14	20
	52	25	50	18	7	
	104	24	37	22	8	9
Blunt	2.6	78	22			
	5.2	75	25			
	10.4	72	28			
	26	97	3			
	52	87	11	2		

Plasmid pUR222 DNA that had been cut by either *Eco*RI (5′ overhang), *Pst* I (3′ overhang), or *Hin*dII (blunt ends) was incubated at the indicated concentrations with RecA in 2 mM $MgCl_2$/0.6 mM ATP[γ-S]/20 mM Hepes, pH 7.5, for 3 hr at 37°C. DNA–protein complexes were fixed and prepared for electron microscopy (22), fields of molecules were photographed, and the lengths of more than 100 filaments were measured at random.

mechanism of dsDNA break repair in *E. coli*, it requires an active *recA* gene (13), other SOS-inducible proteins (28) including the *recN* product (29), and greater than one genome per cell (28). These results have led to models in which dsDNA breaks are repaired through strand-exchange reactions (29–31). However, if the DNA ends in end-joined filaments were able to be covalently joined, this might provide an alternative pathway for the repair of dsDNA breaks. To address this possibility, end-joined filaments containing either dsDNA or ssDNA were incubated with phage T4 or *E. coli* DNA ligase as described in *Materials and Methods*. No covalent joining of either linear ssDNA or linear dsDNA was observed under the conditions tested (data not shown). The apparent inaccessibility to ligase observed for the filaments formed along dsDNA in the presence of Mg^{2+} and ATP[γ-S] was not surprising, due to the very tight protein binding and distortion of the DNA helix present in these complexes. It is possible that other proteins, cofactors, or conditions not tested here would allow direct ligation of the DNA in end-joined filaments.

Table 2. End-joining of filaments containing dsDNA with noncomplementary ends

DNA ends		% multimers		
Structure	pmol/ml	pUR222	pBR322	pUR222/pBR322
*Eco*RI/*Pst* I	2.6	6	2	0
	10.4	2	1	2
	28	50	10	0
	56	52	10	0
*Eco*RI/*Sal* I	2.6	16	2	2
	10.4	16	10	5
	28	46	15	4
	56	39	15	0

Plasmid pBR322 DNA (linearized by *Eco*RI) was incubated with pUR222 DNA [cleaved by either *Pst* I or *Sal* I (5′ overhang, noncomplementary to *Eco*RI ends)] and RecA exactly as described in the legend to Table 1. Filaments containing plasmid pUR222 DNA are 1.3 μm long, whereas filaments containing pBR322 are 2.1 μm long and thus can be distinguished easily.

Table 3. End-joining of filaments containing ssDNA

Cofactors					Filaments		
ATP	dATP	Mg^{2+}	Mn^{2+}	SSB	% fully RecA-covered	% circular	Linear dimers
+	−	+	−	+	25–60	20–50	+
+	−	+	−	−	75–85	40–60	+
−	+	+	−	+	10–30	5–15	−
−	+	+	−	−	80–90	30–60	+
+	−	−	+	+	3–15	<1	−
+	−	−	+	−		Aggregated	
−	+	−	+	+	5–15	<1	−
−	+	−	+	−		Aggregated	
(ATP[γ-S])		+	−	−		Aggregated	

RecA was assembled onto M13 mp7 ssDNA in the presence of various cofactors. DNA–protein complexes were fixed and prepared for electron microscopy, random fields of molecules were photographed and scored, and the filament lengths were measured. Values are from at least three experiments under each set of conditions.

Although Rusche *et al.* (32) have shown that RecA stimulates ligation of dsDNA by T4 ligase in the presence of Mg^{2+} and ATP, that reaction is different from the ones characterized here. Using their conditions we have found that RecA does not bind cooperatively to dsDNA; as visualized by electron microscopy, only rare discrete protein "balls" are present along the DNA (unpublished observation). This argues that the RecA protein in their experiments stimulated ligation by acting as a volume-exclusion agent (33, 34) rather than by specifically juxtaposing the DNA ends. Whatever the detailed mechanism is, it seems that repair of dsDNA breaks would be facilitated if the severed DNA ends were held together. In the cell, an end-joining activity could juxtapose broken DNA ends, properly positioning them for subsequent recombinational pairing, ligation, or other repair processes.

(*ii*) Although RecA protein is known to be involved in the protection of DNA ends from exonucleolytic degradation following damage (12), the mechanism responsible for this is not known. It has been proposed that protection results from RecA binding to the damaged DNA (35), but *in vitro* studies trying to demonstrate this have been inconclusive (36, 37). The apparent inaccessibility of the DNA ends in end-joined filaments to ligases argues that end-joining may provide greater protection from exonuclease degradation than does RecA protein binding alone.

Although ATP[γ-S] (which is not present *in vivo*) was required to obtain end-joining of filaments containing dsDNA *in vitro*, other factors might facilitate this activity *in vivo*. In addition, most DNA ends may not remain double-stranded following dsDNA breaks *in vivo*. Instead, due to the action of the RecBC nuclease, other exonucleases, and helicases, large single-stranded regions may be generated at the breaks. We have shown here that two dsDNA molecules containing long ssDNA tails can be end-joined provided that both 5′ and 3′ ends are available. Thus, if end-joining occurs *in vivo*, it might predominantly involve ssDNA ends.

Possible Functions of End-Joining in Other Systems. In this paper we have described conditions under which RecA protein filaments containing linear DNA are joined together end-to-end, juxtaposing the DNA ends. In work to be reported elsewhere, it has been found that the phage T4 UvsX protein (another recombinational scaffolding protein; ref. 11) also promotes end-joining, and juxtaposes dsDNA ends in the presence of Mg^{2+} and ATP (L. D. Harris and J.G., unpublished observations). These results suggest that end-joining may be a common property of this class of DNA binding proteins. Such proteins may also be present in eukaryotic cells. Indeed, a DNA binding protein in *Xenopus* has been

described (38) that stimulates ligation of linear dsDNA, but it is not known whether this stimulation is due to random DNA aggregation or end-joining of nucleoprotein complexes. In addition, it is known that after transfection into cultured mammalian cells, linear plasmid DNA is rapidly converted into long concatemers (39). Although the mechanism responsible for this is not known, proteins with end-joining activities might well be involved.

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